Technique for the observation of early morphological changes during ectomycorrhiza formation

J. ANDRÉ FORTIN[1] AND YVES PICHÉ
Department of Ecology and Pedology, Faculty of Forestry and Geodesy, Laval University, Ste-Foy, P.Q., Canada G1K 7P4
AND
MAURICE LALONDE
Charles F. Kettering Research Laboratory, 150 East South College Street, Yellow Springs, OH, U.S.A. 45387

Received June 19, 1979

FORTIN, J. A., Y. PICHÉ, and M. LALONDE. 1980. Technique for the observation of early morphological changes during ectomycorrhiza formation. Can. J. Bot. **58**: 361–365.

Flat, transparent polyester growth pouches were used for synthesis of ectomycorrhizae on *Pinus strobus* seedlings. Typical ectomycorrhizae with mantle and Hartig net were obtained within 5 days after inoculation with *Pisolithus tinctorius*. An extensive extramatrical network of hyphae and hyphal strands could be observed within 15 days after ectomycorrhizae formation. The process was somewhat slower with *Cenococcum graniforme*. Other proven ectomycorrhizal fungi on *P. strobus* were unsuccessful in forming ectomycorrhizae under conditions used in these experiments.

FORTIN, J. A., Y. PICHÉ et M. LALONDE. 1980. Technique for the observation of early morphological changes during ectomycorrhiza formation. Can. J. Bot. **58**: 361–365.

Des ectomycorrhizes se sont formées sur des plantules de *Pinus strobus* cultivées en sachets de croissance plats et transparents, faits de polyester, après inoculation avec le *Pisolithus tinctorius*. Vers le 5ième jour après l-inoculation, le manchon et le réseau de Hartig apparaissent; les hyphes et les rhizomorphes forment un réseau extensif dans les 15 jours qui suivent la formation des premières mycorrhizes. Le processus est plus lent avec le *Cenococcum graniforme*. D 'autres champignons ectomycorrhiziens éprouvés du *P. strobus* n'ont pas réussi à former d'ectomycorrhizes avec les conditions expérimentales utilisées ici.

Introduction

Several authors have examined the external morphology (Boullard 1968; Dominik 1969; Zak 1973), as well as the cytology and ultrastructure (Chilvers 1968; Scannerini 1968; Von Hofsten 1969; Marks and Foster 1973; Kiffer 1974; Strullu 1976), of ectomycorrhizae. Although the anatomy of mature ectomycorrhizae has been documented by these authors, none of their observations showed the initial stages of the colonization process, vital linkages established between the cell walls of host and the fungi, or the behavior of the extramatrical mycelium before and after infection.

Studying analogous questions with an actinorhizal association, Lalonde (1979) adapted the growth pouch technique already widely used for the study of legume nodulation (Weavers and Frederick 1972). This technique permitted accurate evaluation of early phases of the colonization process by *Frankia* spp.

The potential use of this method for the study of ectomycorrhizae is obvious. The purpose of this study was to determine its application in research on ectomycorrhizae of pine.

Materials and methods

Seeds of *Pinus strobus* L. were surface sterilized, germinated in Petri dishes, and grown individually under aseptic conditions in test tubes for 30 days. Surface sterilization and germination of seeds, as well as growing conditions for the seedlings before their transfer into pouches, were performed as previously described (Fortin and Piché 1979).

Fifteen millilitres of modified Melin–Norkrans (MNM) mineral nutrient solution (Marx and Bryan 1975) was added to each pouch. Also, a piece of glass tube (2 mm inner diameter, 4 mm outer diameter, and 200 mm long) was placed along the side of each pouch. The tube was used to add nutrient solution and water to the roots through a syringe, without disturbing the root system.

Seedlings were transplanted into the pouches after tap roots were about 10 cm long. The root system was laid directly onto the paper pad. The mouth of each bag was closed with three paper clips to reduce evaporation and contamination.

Before placing the seedling pouches in growth chambers, the level of the nutrient solution in each bag was marked with ink on the outside of the pouch so that nutrient solution levels could be adjusted as needed. In the growth chamber, seedling pouches were hung from wires using clothes pins (Fig. 1) and submitted to the same temperature, light, and humidity conditions as those used for the aseptic production of the seedlings (Fortin and Piché 1979).

The solution level in each pouch was adjusted every 2 days to the ink mark with distilled water, and 5 mL of MNM mineral solution was added every 2 weeks after inoculation.

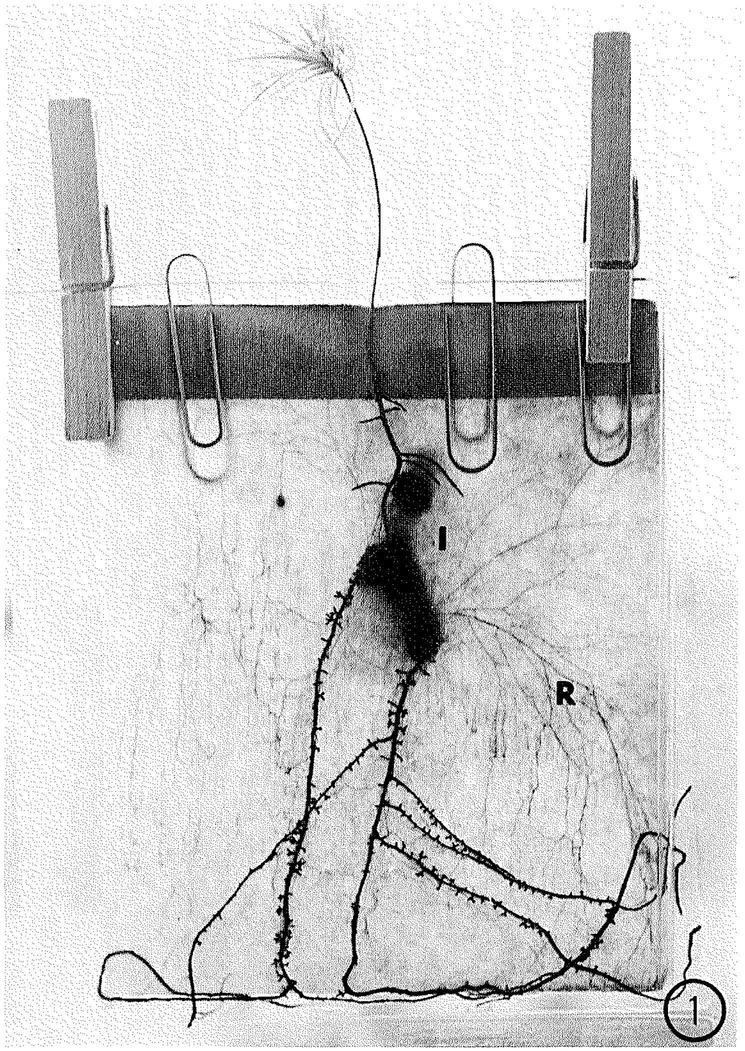
Sixteen days after transfer into the pouches some long lateral roots initiated the formation of short roots. These were inoculated with 7.5 mm diameter circular plugs of complete MNM,

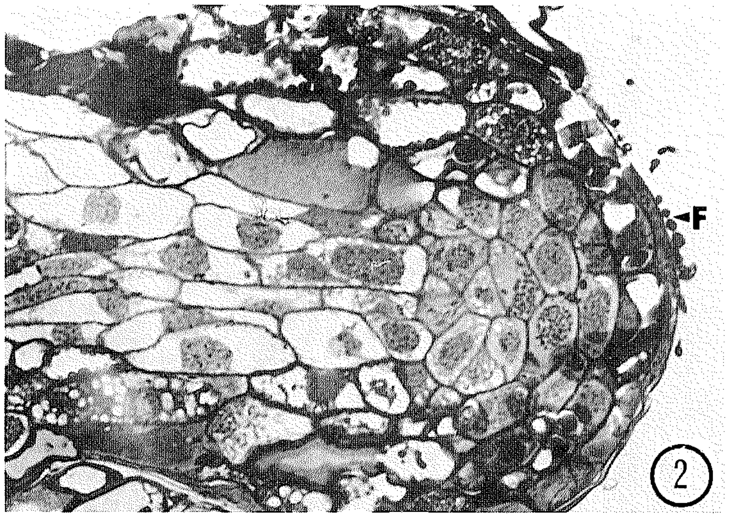
---

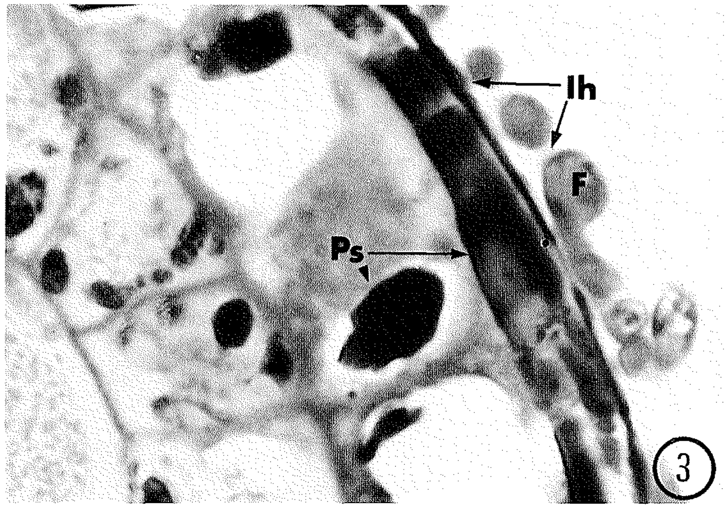
[1] Supported by the Natural Sciences and Engineering Research Council Canada grant No. A-3235.

0008-4026/80/030361-05$01.00/0

©1980 National Research Council of Canada/Conseil national de recherches du Canada

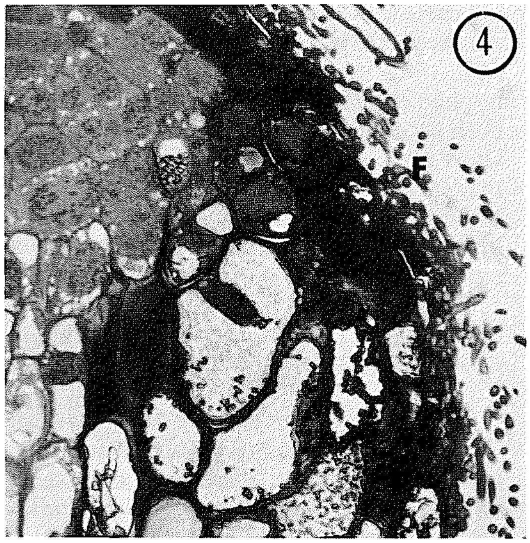


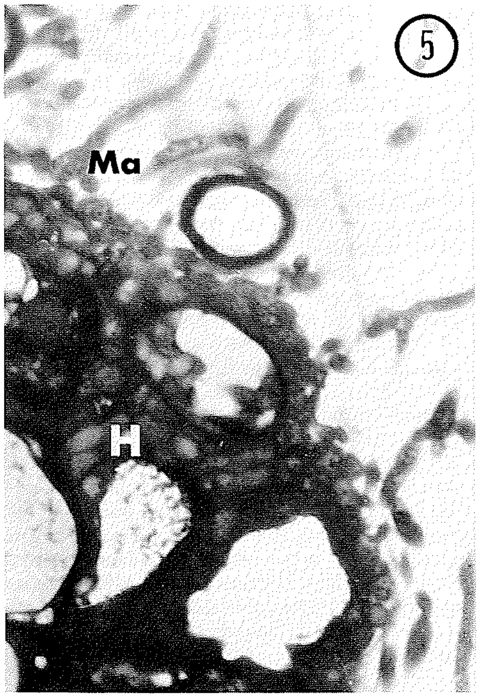


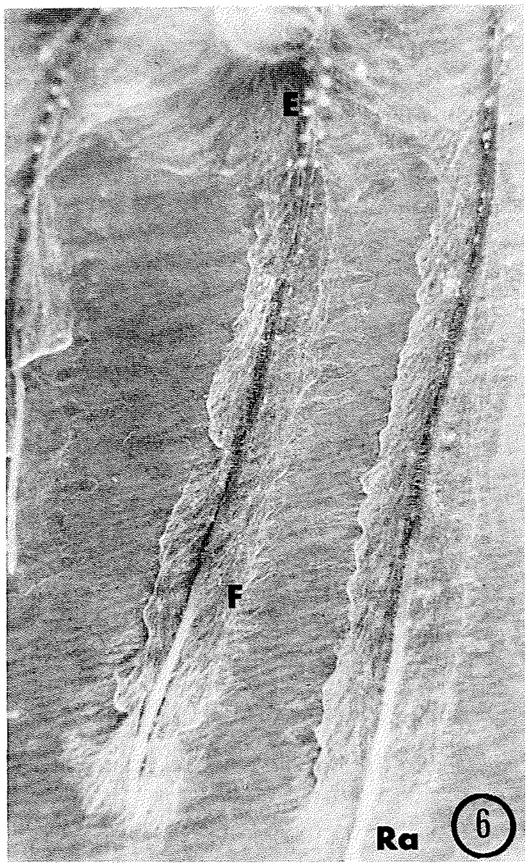


Abbreviations used: E, ectomycorrhizae; F, fungal hyphae; H, Hartig net; Ma, mantle: My, extramatrical mycelium: I, inoculum; Ih, interhyphal material; R, hyphal strands; Ra, root apex; Ps, phenolic substances.

Fig. 1. *Pinus strobus* seedling and plug of *Pisolithus tinctorius* inoculum (I) in the growth pouch. Note the dichotomous branches and the distribution of the extramatrical mycelium forming hyphal strands (R). Fig. 2. Relationship between fungal hyphae (F) and a short root primordium 1 day after inoculation with *P. tinctorius* (light microscope ×125). Fig. 3. Enlargement of Fig. 2 showing details of the relationship between fungal hyphae (F) and external cells of a short root primordium showing phenolic substances (Ps) in epidermal and subepidermal cells as well as interhyphal material (Ih) (light microscope × 1250).







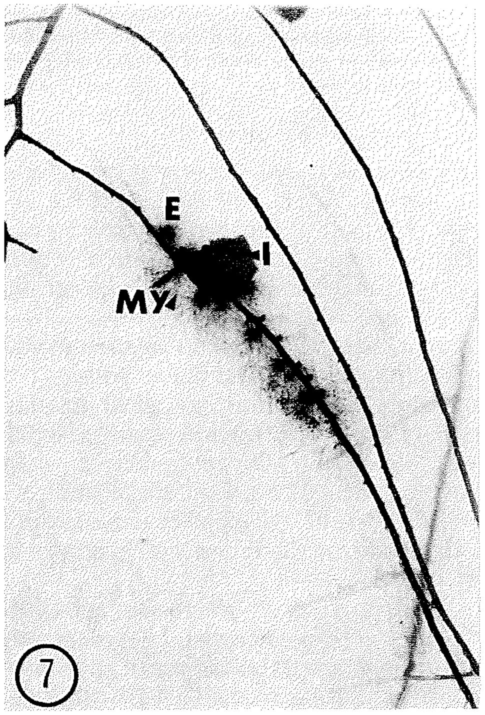


FIG. 4. Relationship between fungal hyphae (F) and a short root primordium 5 days after inoculation with *P. tinctorius* (light microscope × 125). FIG. 5. Detail of the Hartig net (H) (5 days after inoculation; light microscope × 312.5). FIG. 6. Behavior of *P. tinctorius* mycelium growing acropetally and parallel to the long lateral roots, showing concomitant growth of fungal hyphae (F) and root apex (Ra) (×2). FIG. 7. Cluster of ectomycorrhizae (E) formed on *P. strobus* root with *C. graniforme* showing extramatrical mycelium (My) radiating from the short roots. Note the absence of hyphal strands (×2).

containing actively growing mycelium of the test mycorrhizal fungus. The plugs were cut from the edge of a 2- to 3-week-old colony cultivated as previously described (Fortin 1967). The plugs, however, were first incubated on freshly prepared complete MNM agar, mycelium up, in order to permit the regeneration of hyphae at the upper periphery of the plugs. Seven days later, the plugs were transferred into the pouches and placed on the paper pad 3–5 mm from a short root primordium.

The following ectomycorrhizal fungi were used as inoculum: *Pisolithus tinctorius* (Pers.) Coker and Couch, a 1976 isolate obtained from a young basidiocarp in the Arcachon-Landes area of France by D. Mousain (Montpellier); *Cenococcum graniforme* (Sow) Ferd. and Winge, a 1967 isolate obtained from a surface sterilized *Picea mariana* ectomycorrhiza in southern Quebec by C. Richard; *Leccinum chromapes* (Frost) Sing., *Suillus granulatus* (L. ex. Fr.) O. Kuntze, and *Suillus tomentosus* (Kauffm) Sing. Snell and Dick, three isolates obtained from young sporophores in southern Quebec in 1977; and finally *Thelephora terrestris* (Ehrh.) Fr., an isolate obtained by C. Richard in 1971. Before 1977 the isolates were kept on MNM medium enriched with 1% malt extract, at 4°C under mineral oil. Since September 1977 they were transferred once a month on MNM medium enriched with 1% malt. All of these fungi formed ectomycorrhizae readily in our laboratory when Hacskaylo's technique (1953) as modified by Marx and Zak (1965) was used.

Seedlings were periodically collected and root material was fixed, at 5°C with 3% glutaraldehyde buffered at pH 7.3 with Sorensen's buffer, for 16 h followed by a post fixation in 2% osmium tetroxide for 2 h. Fixed specimens were dehydrated in increasing ethanol concentrations and embedded in Epon 812. Sections were cut 2–6 μm thick, picked up with a loop containing a film of water, transferred onto clean glass slides, and allowed to dry on a hot plate for at least 5 min at 60°C prior to staining with methylene blue for 1 min.

Results

Only *P. tinctorius* and *C. graniforme* formed ectomycorrhizae. Aerial mycelia of these fungi were easily observed at the upper periphery of the plugs during preinoculation incubation whereas with the other fungi the hyphae tended to grow on the lateral surface of the plug.

The contact of the *P. tinctorius* mycelium with a short root primordium took place within 1 day (Figs. 2, 3) and the early morphological manifestation of ectomycorrhiza formation occurred within 5 days. At this time the Hartig net and fungus mantle were evident (Figs. 4, 5). Epidermal cells were necrotic and within 8–9 days after inoculation the apex of mycorrhizae flattened and dichotomous branching began.

A second feature of *P. tinctorius* ectomycorrhizae was the extensive acropetal development of the external mycelium at the surface of and 3 mm on either side of the long root. The growth rate of hyphal tips often paralleled that of elongating long roots (Fig. 6). Ectomycorrhizal development did not occur in lateral root cortex.

Six weeks after inoculation extensive networks of hyphal strands were formed, reaching almost all parts of the pads (Fig. 1).

Formation of ectomycorrhizae by *C. graniforme* occurred within 2 weeks after inoculation (Fig. 7). Following the first contact with the root, sparse hyphae developed on the surface of the long root, both acropetally and basipetally. After contacting a short root, the mycelium developed intensively at the surface. These colonized roots appeared to grow faster than the controls and they eventually branched dichotomously. Shortly thereafter, the external mycelium grew away from the root.

The other fungi have consistently failed to colonize root systems and to produce ectomycorrhizae using this method.

Discussion

The use of polyester growth pouches is a useful tool for the study of ectomycorrhizae. With *P. tinctorius* the first morphological indications of ectomycorrhizae took place within 3 days and full anatomical evidence of ectomycorrhiza formation was obtained within 5 days. With *C. graniforme* the process was somewhat slower.

The pouch method also permitted observation of the development of external mycelium before and after the formation of the ectomycorrhizae. The observations suggest that the short roots are exuding substances stimulating the growth of *P. tinctorius* and *C. graniforme*. In the case of *P. tinctorius*, the observations also suggested that the growth of the fungus is kept at a distance down the root as if an inhibitor(s) was acting simultaneously. The concomitant growth of the long root apex and the mycelium of *P. tinctorius* (Fig. 6) demonstrates that a close and organized relationship between the host plant and its fungal symbiont can exist even before the formation of ectomycorrhizae.

The reasons why the other mycorrhizal fungi failed to colonize the roots and to form ectomycorrhizae are not obvious; moisture level and aeration conditions as well as exposure to light could possibly be related to these repeated failures. The age and the history of the isolates could also be possible causes of failure.

Acknowledgements

The authors express their gratitude to Dr. Jean Lafontaine for assistance in preparing microscopic observations, and to Marthe Simard for assistance in preparing the manuscript.

BOULLARD, B. 1968. Les mycorrhizes. *Edited by* Masson and Cie. Paris.

CHILVERS, G. A. 1968. Low power electron microscopy of the root cap region of eucalypt mycorrhizas. New Phytol. **67**: 663.

DOMINIK, T. 1969. Key to ectotrophic mycorrhizae. Folia For. Pol. Ser. A, **15**: 309–328.

FORTIN, J. A. 1967. Action inhibitrice de l'acide 3-indolylacétique sur la croissance de quelques basidiomycètes mycorrhizateurs. Physiol. Plant. **20**: 528–532.

FORTIN, J. A., and Y. PICHÉ. 1979. Cultivation of *Pinus strobus* root-hypocotyl explants for synthesis of ectomycorrhizae. New Phytol. **81**: 109–119.

HACSKAYLO, E. 1953. Pure culture synthesis of pine mycorrhizae in terralite. Mycologia, **45**: 971–975.

KIFFER, E. 1974. Etude des champignons mycorrhiziens et de quelques autres souches associées à l'Epicéa en Lorraine. Thèse doctorat de spécialité, Université de Nancy.

LALONDE, M. 1979. Techniques and observations of the nitrogen-fixing *Alnus* root nodule symbiosis. *In* Recent advances in biological nitrogen fixation. *Edited by* N. S. Subba Rao. Oxford and IBH Publication, New Delhi. In press.

MARKS, G. C., and R. C. FOSTER. 1973. Structure, morphogenesis and ultrastructure of ectomycorrhizae. *In* Ectomycorrhizae. *Edited by* G. C. Marks and T. T. Kozlowski. Academic Press, New York, NY. pp. 1–41.

MARX, D. H., and B. ZAK. 1965. Effect of pH on mycorrhizal formation of Slash Pine in asepticulture. For. Sci. **11**: 66–75.

MARX, D. H., and W. C. BRYAN. 1975. Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius*. For. Sci. **21**: 245–254.

SCANNERINI, S. 1968. Sull ultrastructure delle ectomycorrhize II. Ultrastruttura di una micorriza di Ascomycete "*Tuber albidum*" × *Pinus strobus*. Allionia, **14**: 77–95.

STRULLU, G. D. 1976. Recherches de biologie et de microbiologie forestières. Etude des relations nutrition–dévelopment et cytologie des mycorrhizes chez le Douglas (*Pseudotsuga menziesii* Mirb.) et les Abiétacées. Thèse doctorat en sciences naturelles.

VON HOFSTEN, A. 1969. The ultrastructure of mycorrhiza. I. Ectotrophic and ectendotrophic mycorrhiza of *Pinus silvestris*. Sven. Bot. Tidskr. **63**: 455–464.

WEAVERS, R. W., and L. R. FREDERICK. 1972. A new technique for most probable number counts of Rhizobia. Plant Soil, **36**: 219–222.

ZAK, B. 1973. Classification of ectomycorrhizae. *In* Ectomycorrhizae. *Edited by* G. C. Marks and T. T. Kozlowski. Academic Press, New York, NY. pp. 43–78.